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The Disease Resistance Signaling Components *EDS1* and *PAD4* Are Essential Regulators of the Cell Death Pathway Controlled by *LSD1* in Arabidopsis

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Specific recognition of pathogens is mediated by plant disease resistance (*R*) genes and translated into a successful defense response. The extent of associated hypersensitive cell death varies from none to an area encompassing cells surrounding an infection site, depending on the *R* gene activated. We constructed double mutants in Arabidopsis between positive regulators of *R* function and a negative regulator of cell death, *LSD1*, to address whether genes required for normal *R* function also regulate the runaway cell death observed in *lsd1* mutants. We report here that *EDS1* and *PAD4*, two signaling genes that mediate some but not all *R* responses, also are required for runaway cell death in the *lsd1* mutant. Importantly, this novel function of *EDS1* and *PAD4* is operative when runaway cell death in *lsd1* is initiated through an *R* gene that does not require *EDS1* or *PAD4* for disease resistance. *NDR1*, another component of *R* signaling, also contributes to the control of plant cell death. The roles of *EDS1* and *PAD4* in regulating *lsd1* runaway cell death are related to the interpretation of reactive oxygen intermediate–derived signals at infection sites. We further demonstrate that the fate of superoxide at infection sites is different from that observed at the leading margins of runaway cell death lesions in *lsd1* mutants.

INTRODUCTION

Plants have evolved mechanisms to detect and respond effectively to most pathogens. Analyses of genetic variation in plant responses to pathogens have identified corresponding gene pairs (resistance or *R* genes in the plant and avirulence or *avr* genes in the pathogen) that mediate recognition and cause induction of plant resistance (Staskawicz et al., 1995). These local plant defenses are usually, although not invariably, associated with a form of programmed plant cell death known as the hypersensitive response (HR). The HR can lead to cell death surrounding the infection site (Holub et al., 1994). Localized necrosis also can induce a plant response called systemic acquired resistance, which heightens defenses in uninoculated tissues against a broad spectrum of pathogens (Yang et al., 1997; McDowell and Dangl, 2000).

One of the earliest biochemical changes associated with the HR is an oxidative burst producing reactive oxygen intermediates (ROI), including superoxide anion ($O_2^{-}\cdot$) as a proximal component, which can be dismutated rapidly to hydrogen peroxide (H_2O_2) (Lamb and Dixon, 1997; Bolwell, 1999; Grant and Loake, 2000). These may serve both as antimicrobial agents and as signaling molecules in local and systemic plant resistance. Nitric oxide (NO), a redox-active molecule with a critical role in the activation of mammalian defense responses (Schmidt and Walter, 1994), also functions as an important signal in plant resistance against pathogens (Delledonne et al., 1998; Durner et al., 1998). Salicylic acid (SA) accumulates in plant tissue responding to pathogen infection and is essential for the induction of systemic acquired resistance as well as being required for some *R* gene–mediated responses, at least in Arabidopsis and tobacco (Gaffney et al., 1993; Delaney et al., 1994; Mur et al., 1997). Recent results suggest that the balance and cooperation between NO, ROI, and SA produced early in the plant resistance response is required for the full expression of the HR (Delledonne et al., 1998, 2001; Klessig et al., 2000). However, little is known about the sequence of events that determines local plant resistance. Also unclear is whether signals are transduced from an infection focus to first initiate, and then dampen, the HR.

Arabidopsis is the key genetic system with which to unravel disease resistance pathways (Glazebrook, 1999; Feys and Parker, 2000). Arabidopsis *R* genes have been cloned that confer specific recognition of viral, bacterial, and oomycete pathogens (Parker et al., 2000). Their products

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belong to the most prevalent R protein class identified in a range of plant species that contains a central nucleotide binding (NB) domain and varying numbers of C-terminal leucine-rich repeats (LRRs) (Jones, 2000). NB-LRR proteins were further categorized into those with a coiled-coil (CC) motif at their N termini and those that have N-terminal (TIR) similarity to the cytoplasmic domains of human and Drosophila Toll-like receptors (Jones, 2000).

Mutational analyses in Arabidopsis uncovered genes required as positive regulators of basal defense (Glazebrook, 1999; Feys and Parker, 2000). *EDS1* is a necessary component of *RPP1*- and *RPP4*-specified resistance to the oomycete pathogen *Peronospora parasitica* (*Pp*) (Parker et al., 1996; Aarts et al., 1998) and is more generally required for resistance mediated by several tested Arabidopsis *R* genes encoding TIR-NB-LRR proteins (Aarts et al., 1998). However, *EDS1* is not required for resistance conferred by any of the tested CC-NB-LRR *R* genes (Aarts et al., 1998). Many, but not all, CC-NB-LRR *R* genes examined are dependent on *NDR1*, a gene identified through mutational analysis of *RPM1*-mediated resistance to the bacterial pathogen *Pseudomonas syringae* expressing *avrB* (Century et al., 1995). Thus, *EDS1* and *NDR1* differentiate *R* gene–mediated events that may, at least in several cases, be conditioned by particular R protein structural types (for the current exceptions, see McDowell et al., 2000). Furthermore, *ndr1* mutant plants retain an HR initiated by two *R* genes, *RPM1* and *RPS5*, even though they fail to prevent bacterial growth, suggesting that resistance and HR are separable (Century et al., 1995). *EDS1* encodes a 72-kD lipase-like protein that operates upstream of SA-mediated defenses (Falk et al., 1999), whereas *NDR1* encodes a 25-kD protein that has two putative membrane attachment domains (Century et al., 1997).

Mutational screens in Arabidopsis identified several other plant defense signaling genes that are components of SA signaling in the plant response against pathogens. For example, *PAD4* (Glazebrook et al., 1997; Zhou et al., 1998) and *SID1*/*EDS5* and *SID2*/*EDS16* (Rogers and Ausubel, 1997; Nawrath and Métraux, 1999) function upstream of SA accumulation, whereas *NPR1*/*NIM1* is an important regulator of responses downstream of SA (Cao et al., 1994; Delaney et al., 1995). Significantly, *PAD4* encodes a lipase-like protein with catalytic motifs identical to EDS1 (Jirage et al., 1999). *EDS1* and *PAD4* operate upstream of pathogen-induced SA accumulation, yet their expression can be enhanced by exogenous applications of SA. This finding reinforces evidence of an SA-associated positive feedback loop that may potentiate plant defense (Shirasu et al., 1997; Falk et al., 1999; Jirage et al., 1999). The *pad4* mutation affects the same spectrum of *R* gene functions detailed above for *eds1*, but the loss of resistance in *pad4* is typically not as complete as in *eds1* (Glazebrook et al., 1997; Aarts et al., 1998; Feys et al., 2001).

Other Arabidopsis mutations deregulate disease resistance responses and/or HR-like plant cell death responses, suggesting that negative control of plant defense pathways also occurs (Morel and Dangl, 1997). Some of these display a "disease lesion mimic" phenotype that is a feature of several well-characterized crop plant mutants, in which necrotic lesions form spontaneously or can be induced by various biotic or abiotic stresses (Dangl et al., 1996; Büschges et al., 1997; Gray et al., 1997). Importantly, Arabidopsis plants carrying the recessive null *lsd1* allele exhibit normal HR after infection by various incompatible pathogens, but runaway cell death (RCD) is initiated subsequently at the margins of these sites (Dietrich et al., 1994). Spreading lesions in *lsd1* can be induced by provision of $O_2^{-}\cdot$ (Jabs et al., 1996) in uninfected tissues. This, together with observations that $O_2^{-}\cdot$ accumulation precedes lesion formation (Jabs et al., 1996), suggests that LSD1 responds to a superoxide-dependent signal(s) emanating from an infection site. SA possibly potentiates this pathway, because *lsd1* plants are acutely responsive to treatments with SA or chemically active SA analogs (Dietrich et al., 1994; Jabs et al., 1996). Thus, *lsd1* lowers the threshold for both initiation and propagation of plant cell death beyond the HR. *lsd1* plants also exhibit enhanced resistance to several normally virulent pathogens in a prelesioned state (Dietrich et al., 1994). We infer from these null phenotypes that LSD1 negatively regulates a signaling pathway(s) for basal defense and cell death and thereby may contribute to establishing a boundary to the plant HR (Dietrich et al., 1994). *LSD1* encodes a zinc finger protein with homology with GATA-type transcription factors, and it has been suggested that the LSD1 protein functions either to negatively regulate a pro-death pathway component or to activate a repressor of plant cell death (Dietrich et al., 1997).

We constructed double mutant lines between the *eds1*, *pad4*, or *ndr1* mutations and *lsd1* and assessed their effects on RCD and disease resistance phenotypes after pathogen infection, treatment with benzothiodiazole (BTH), a functional SA mimic (Görlach et al., 1996), or a superoxide generator. We demonstrate that *lsd1* does not affect the *eds1*, *pad4*, and *ndr1* pathogen response phenotypes. However, both *EDS1* and *PAD4* are necessary for *lsd1*-conditioned RCD initiated by each tested stimulus. In contrast, *NDR1* is required for RCD in response to superoxide and partially reduces *lsd1* RCD after pathogen inoculation or BTH treatment. The requirement for *EDS1*, *PAD4*, or *NDR1* in *lsd1* RCD is separable from processes associated with the local HR and disease resistance; therefore, it is likely to operate at the level of defense signal potentiation in cells surrounding an infection site.

RESULTS

EDS1 and *PAD4* Are Required for *lsd1* RCD Induced by BTH and Pathogens

We first examined the responses of short-day-grown *eds1*/*lsd1*, *pad4*/*lsd1*, and *ndr1*/*lsd1c* plants to a known inducer of

RCD in *lsd1*, the SA mimic BTH (all mutants used were null alleles; see Methods). As shown in the top row of Figures 1A and 1B, no phenotype was observed in leaves from either wild-type plants or plants with single mutations in *eds1*, *pad4*, or *ndr1*. Leaves from *lsd1* or *lsd1c* plants, in contrast, formed the expected lesions in response to BTH 3 days after treatment. We did not observe any lesions in leaves of plants with double mutations in *eds1*/*lsd1* and *pad4*/*lsd1*, but lesions were observed in leaves of *ndr1*/*lsd1c* plants. However, these lesions were not as extensive as those observed in leaves of *lsd1* plants (Figure 1B). Thus, mutations in *eds1* or *pad4* abolish BTH-induced RCD in *lsd1* plants. Similar results were observed in these plants after treatment with another inducer of *lsd1*-mediated RCD, a shift in growing conditions from short-day to long-day conditions (data not shown).

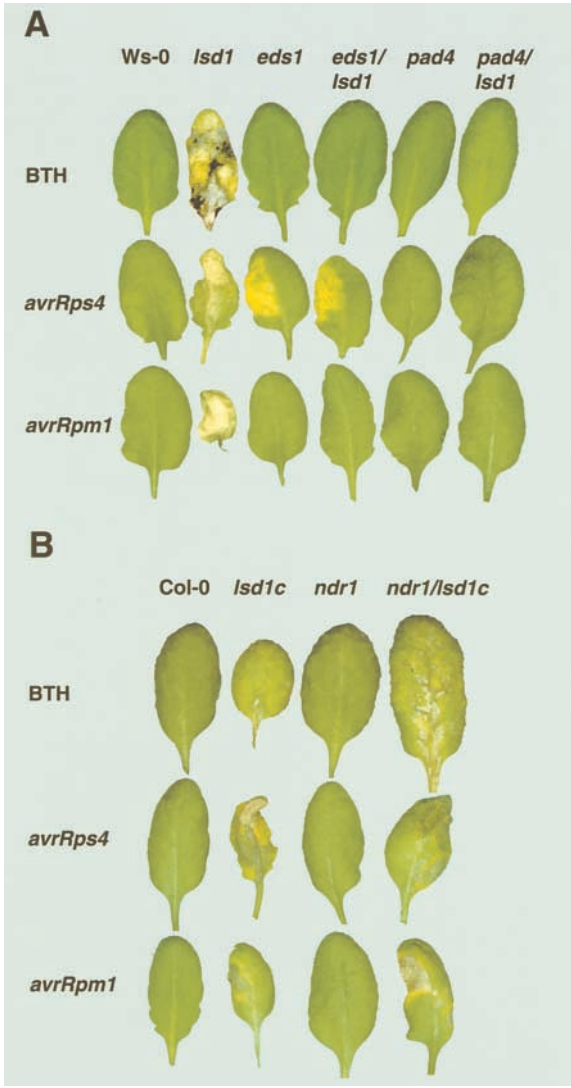

Figure 1. Lesion Phenotypes of Plant Lines after BTH Treatment or Bacterial Pathogen Inoculation.

Leaves of 4-week-old wild-type, single mutant, or double mutant plants were sprayed with 0.35 mM BTH or infiltrated on one side with low titer suspensions (10^5 colony-forming units/mL) of *P. syringae* pv DC3000 expressing *avrRps4* or *avrRpm1*. See Methods for further details. Leaves were photographed after 6 days of incubation, and each leaf is representative of 12 to 15 leaves. All treatments were repeated with similar results.

(A) Phenotypes of plant lines in accession Ws-0.

(B) Phenotypes of plant lines in accession Col-0.

We next assessed the interactions between these double mutant plants and normally avirulent strains of *P. syringae* pv *tomato* (DC3000) expressing either *avrRpm1* or *avrRps4*. The different signaling requirements for *RPM1* and *RPS4* mentioned in the Introduction allowed us to measure the effects of the *eds1*, *pad4*, and *ndr1* mutations on *lsd1*-induced phenotypes in the context of both an intact (resistant) and a defective (susceptible) local plant response by using isogenic *P. syringae* strains differing only in the *avr* gene they express. Plants were infiltrated with low doses of DC3000/*avrRps4* to examine the genetic interactions between *eds1* or *pad4* in combination with *lsd1*. As expected, Wassilewskija (Ws-0) and *lsd1* plants were resistant (Figure 2A). However, Ws-0 plants exhibited no visible phenotype, whereas *lsd1* plants displayed lesions 3 to 4 days postinoculation (DPI) (Figure 1A). In contrast, *eds1* and *eds1*/*lsd1* double mutant plants were susceptible (Figure 2A). Additionally, *eds1* and *eds1*/*lsd1* double mutant plants developed characteristic chlorotic disease symptoms, but no spreading lesions were observed in the *eds1*/*lsd1* double mutant plants (Figure 1A). Plants with mutations in *pad4* or both *pad4* and *lsd1* were intermediate; bacterial growth was ~10-fold less than in plants with mutations in *eds1* (Figure 2A). However, *pad4*/*lsd1* plants did not exhibit chlorosis associated with disease or pathogen-induced lesioning associated with *lsd1* RCD (Figure 1A). Thus, *lsd1* does not influence the susceptibility of *eds1* or *pad4* plants to DC3000/*avrRps4*.

We then challenged plants with low doses of DC3000/*avrRpm1*. Wild-type, *lsd1*, *eds1*, and *pad4* plants responded as expected (see Introduction); all genotypes were resistant, and RCD was visible in *lsd1* leaves (Figures 1A and 2C). *eds1*/*lsd1* and *pad4*/*lsd1* double mutants also were resistant, but, surprisingly, they did not exhibit RCD. To confirm this observation, we infiltrated leaves with levels of DC3000/*avrRpm1* (10^7/mL) that induce an HR 6 to 8 hr after inoculation (Grant et al., 1995). Plants from all genotypes (Ws-0, *lsd1*, *eds1*, *eds1*/*lsd1*, *pad4*, and *pad4*/*lsd1*) exhibited an HR. However, spreading lesions were observed in only *lsd1* plants and not in *eds1*/*lsd1* or *pad4*/*lsd1* (Table 1). Therefore, *EDS1* and *PAD4* are required for *lsd1* RCD. Importantly, the requirement for *EDS1* and *PAD4* in RCD is independent of their signaling functions in *RPS4*-mediated disease resistance and separate from processes controlling *RPM1* resistance.

We also infiltrated *ndr1*/*lsd1c* double mutants with either DC3000/*avrRps4* or DC3000/*avrRpm1*. Columbia (Col-0) and *ndr1* plants were resistant to DC3000/*avrRps4* (Figures

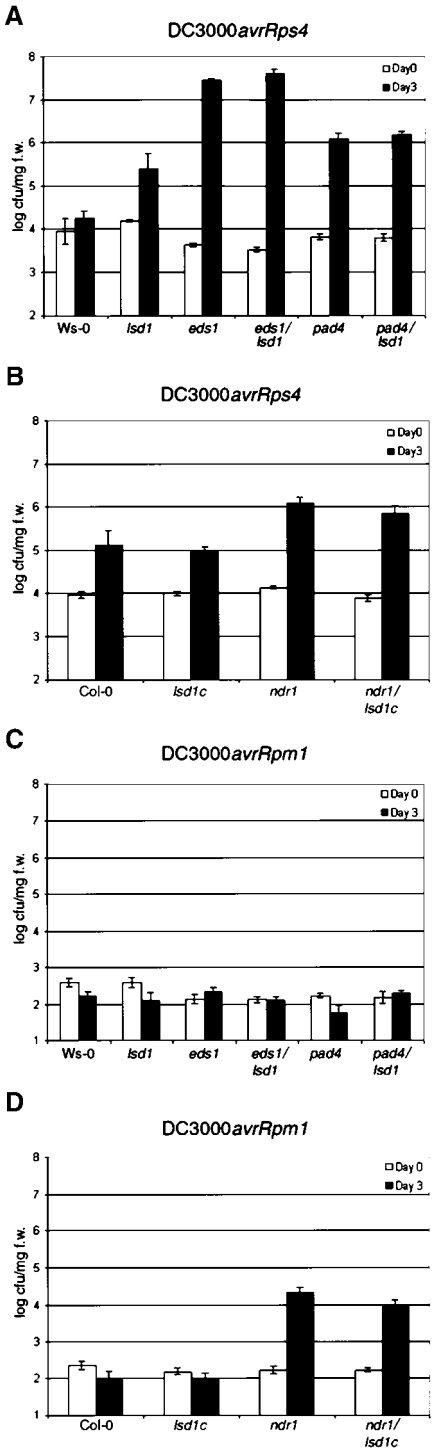

Figure 2. Bacterial Growth in Wild-Type, Single Mutant, and Double Mutant Plants.

Growth of *P. syringae* pv DC3000 expressing *avrRps4* or *avrRpm1* extracted from leaves at 0 (open bars) and 3 (closed bars) days after inoculation (initial titer, 10^5 colony-forming units/mL). Data from Ws-0 accession lines are presented in **(A)** and **(C)**, and data from Col-0 accession lines are presented in **(B)** and **(D)**. Bars represent the mean and ±SD of four independent data points. Similar results were obtained in two independent experiments. cfu, colony-forming units; f.w., fresh weight.

1B and 2B), whereas *ndr1* plants were moderately susceptible to infection by DC3000/*avrRpm1*, consistent with previous analyses (Century et al., 1995) (Figures 1B and 2D). *ndr1/lsd1c* double mutants were resistant to DC3000/*avrRps4* and susceptible to DC3000/*avrRpm1*, as expected. However, we observed a partial suppression of RCD in these plants after inoculation with either bacterial strain (Figure 1B). Therefore, although *ndr1* reduced *lsd1* RCD, the level of reduction did not correlate with the loss of *RPM1* function observed in *ndr1* mutants.

We then examined the responses of the *eds1/lsd1*, *pad4/lsd1*, and *ndr1/lsd1c* mutant lines to normally avirulent isolates of the oomycete pathogen *Pp*. This extends our analysis to an additional pathogen recognized by *R* genes that differ in their signaling requirements, as outlined in the Introduction. As shown in Figures 3A and 3B, *RPP1*-mediated resistance to Noco2 in cotyledons of Ws-0 and *lsd1* is manifested as HR at points of attempted pathogen penetration 6 DPI. At this time, developing RCD is visible in *lsd1* as an enlargement of the trypan blue–stained zone around an infection site (Figure 3A). In contrast, cotyledons of *eds1* and *eds1/lsd1* plants were susceptible to Noco2; we observed extensive mycelial growth as well as asexual sporulation (Figures 3A and 3B). Noco2 inoculation failed to elicit an HR in *eds1/lsd1* plants. *pad4* and *pad4/lsd1* plants were partially susceptible to Noco2; we observed trailing necrosis in response to Noco2 (Figure 3A), suggesting that HR was elicited but not sufficient to fully restrict pathogen growth. There was no RCD in either double mutant. Therefore, EDS1 and PAD4 are required for *lsd1*-mediated RCD, using *Pp* as an RCD inducer.

A similar analysis was performed by inoculating Emoy2 onto cotyledons of Col-0, *lsd1c*, *ndr1*, and *ndr1/lsd1c* plants. *RPP4*-mediated resistance to Emoy2 in Col-0 and *lsd1c* was associated with HR and the initiation of RCD in *lsd1* cotyledons at 6 DPI (Figure 3A). *ndr1* partially suppressed *RPP4*-mediated resistance to Emoy2; we observed an increased frequency of trailing necrosis (Figures 3A and 3B). *lsd1c* plants expressed strong resistance to Emoy2, as shown by an increase in the proportion of HR sites, compared with Col-0 (Figure 3B). Surprisingly, *ndr1/lsd1c* double mutants exhibited an intermediate phenotype (Figure 3B). Therefore, the loss of *LSD1* function enhanced host resistance to *Pp* early in the plant–pathogen interaction independent of *NDR1* and presumably independent of the recognition conferred by *RPP4*.

Table 1. Response Phenotypes of Wild-Type, Single Mutant, and Double-Mutant Lines to Inoculation with Avirulent Bacteria or Treatment with RB

Arabidopsis Lines	*DC3000/avrRps4*			*DC3000/avrRpm1*			RB
	HR	ROI	RCD	HR	ROI	RCD	RCD
Ws-0	+	+	−	+	++	−	−
eds1	−	−	−	+	++	−	−
pad4	+	+	−	+	++	−	−
lsd1	+	++	+	+	+++	+	+
eds1/lsd1	−	−	−	+	+++	−	−
pad4/lsd1	+	+	−	+	++	−	−
Col-0	(+)	(+)	−	+	+	−	−
ndr1	−	−	−	*	++	−	−
lsd1c	(+)	+	+	+	++	+	+
ndr1/lsd1c	−	−	(+)	*	++	(+)	−

Leaves were dipped in suspensions (10^7 colony-forming units/mL) of *P. syringae* pv DC3000 expressing *avrRps4* or *avrRpm1* or treated with a 2-μL droplet of 20 mM RB. Development of the plant HR and accumulation of ROI were scored 24 and 48 hr, and RCD was scored 4 days, after bacterial inoculation. The scores (+), +, ++, and +++ reflect the intensity of staining with lactophenol–trypan blue for the HR and DAB for ROI. They are representative of at least six leaves per treatment. Asterisks denote an expanded, diffuse HR observed in *ndr1* and *ndr1/lsd1c* plants. Pathogen inoculations were repeated twice with similar results. Leaves were scored for RCD 5 days after rose bengal application. Similar results were obtained in four independent experiments using 10 leaves per plant line.

We next examined the responses of leaves from mature (3- to 4-week-old) plants to inoculations of *Pp*. This analysis allowed us to relate events associated with the plant HR to the initiation and spread of *lsd1*-conditioned RCD. As shown in Figures 4A and 4B, discrete necrotic flecks formed on leaves of Ws-0 or Col-0 in the area of incompatible *Pp* inoculation. Leaves of *lsd1* produced lesions that spread from the site of the localized resistance response (Figures 4A and 4B). In contrast, asexual sporulation of *Pp* was observed on infected *eds1* and *eds1/lsd1* leaves at 6 DPI, and no necrosis was observed. Leaves of *pad4* and *pad4/lsd1* plants supported some pathogen growth that was accompanied by trailing necrosis (Figure 4A). These results mirror those observed on cotyledons and further support the requirement for *EDS1* and *PAD4* for *lsd1*-mediated RCD. Leaves of *ndr1* and Col-0 responded in a similar manner to inoculation of *Pp* Emoy2, although the area of plant tissue undergoing an HR was marginally larger in *ndr1* than in Col-0 (Figure 4B; see also Figure 4D). The RCD was severely reduced in *ndr1/lsd1c* compared with that in *lsd1c* (Figure 4B). Interestingly, the rate of initial lesion formation at the boundary of the HR was similar in *lsd1c* and *ndr1/lsd1c* (data not shown). However, by 3 to 4 DPI, lesions in *ndr1/lsd1c* ceased to expand, whereas in *lsd1c* they progressed and consumed the entire leaf by ~6 DPI (Figures 4B and 4D).

EDS1 and *PAD4* Function in RCD Is Downstream of, or Independent from, the Local HR and Associated ROI Accumulation

An oxidative burst giving rise to local ROI accumulation is an early event associated with the plant HR (Bestwick et al., 1997; Shirasu et al., 1997; Thordal-Christensen et al., 1997). Also, $O_2^{-}\cdot$ is necessary and sufficient for *lsd1* RCD (Jabs et al., 1996). We examined the production of ROI in wild-type and mutant plants at the point of pathogen penetration to determine whether the effects of *eds1*, *pad4*, or *ndr1* on *lsd1*-induced lesion propagation could be related to deficiencies in early ROI accumulation during the HR. Excised leaves were dipped in a solution of 3,3-diaminobenzidine (DAB) to visualize H_2O_2 and then inoculated with a 10-μL droplet of avirulent *Pp* conidia, or they were dipped into suspensions of *P. syringae*. DAB polymerizes as a brown precipitate on contact with H_2O_2 in the presence of peroxidase (Shirasu et al., 1997; Thordal-Christensen et al., 1997), thus providing a useful marker for total peroxide accumulation.

The results from this analysis are shown in Figures 4C and 4D and are summarized in Table 1. A plant oxidative burst producing detectable local concentrations of H_2O_2 was observed only in plant genotypes undergoing an HR. Thus, *eds1* (and *eds1/lsd1*) plants challenged with *Pp* Noco2 or DC3000/*avrRps4*, in which resistance is suppressed, failed to elicit an oxidative burst or an HR. *pad4* (and *pad4/lsd1*) plants generated high levels of H_2O_2 and also developed either trailing necrosis or an HR, depending on the pathogen challenge. We conclude that *EDS1* activity is required for the oxidative burst in *EDS1*-dependent *R* gene–mediated responses, whereas *PAD4* functions either downstream or independently of ROI accumulation in the same responses. Both *eds1* and *pad4* plants produced a wild-type *RPM1*-mediated oxidative burst and HR after challenge with DC3000/*avrRpm1* (Table 1). Thus, neither *EDS1* nor *PAD4* is required for the HR-associated oxidative burst in this *EDS1*-independent pathway. Yet, both are required for RCD in any of the tested contexts. Significantly, therefore, the requirements for *EDS1* and *PAD4* during *lsd1*-dependent RCD are unrelated to their effects on local *R* gene–mediated HR. We conclude from these results that *EDS1* and *PAD4* provide necessary signaling functions leading to *lsd1* RCD that are either downstream or independent of the local HR and associated ROI accumulation.

Interestingly, *ndr1* exhibited a reduction in the intensity of HR-associated DAB staining compared with that of Col-0 in response to *Pp* Emoy2, even though more host cells died, as measured by trypan blue (Figure 4D). This is in contrast to the enhanced H_2O_2 accumulation in *lsd1* and *lsd1c* (Figures 4C and 4D). The response of *ndr1/lsd1c* plants was intermediate between that of *ndr1* and *lsd1c* alone, and the RCD boundary was less well defined than it was in wild-type plants (Figure 4D). These data suggest that the reduced ROI production in *ndr1* may be responsible for the attenuated RCD observed in the *ndr1/lsd1c* double mutant.

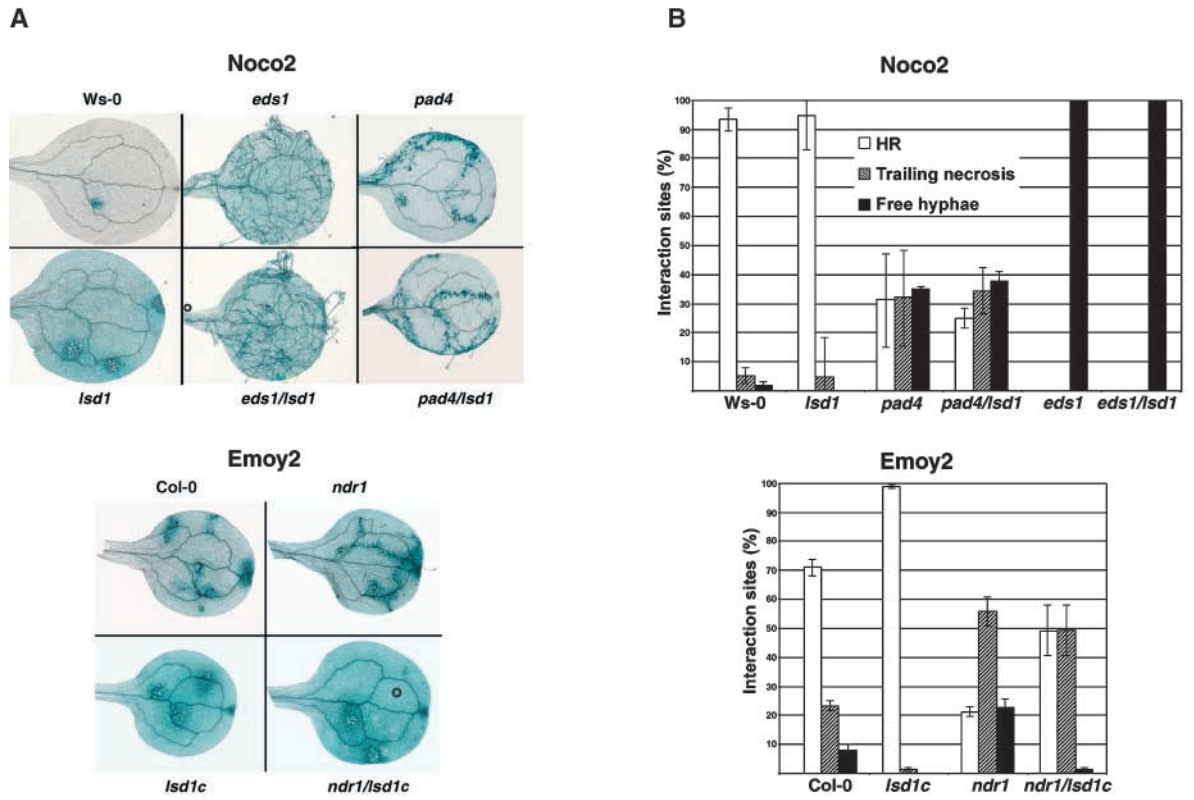

Figure 3. Infection Phenotypes of Plant Cotyledons Inoculated with *Pp* Isolates Noco2 and Emoy2.

(A) Cotyledons of 10-day-old seedlings were inoculated with Noco2 or Emoy2 (5×10^4 spores/mL) and stained with lactophenol–trypan blue at 6 DPI to reveal *Pp* mycelium and dead plant cells.

(B) Trypan blue–stained cotyledons were harvested at 6 DPI, and individual plant–pathogen interaction sites were categorized as HR, trailing necrosis, or free mycelium. The percentage of each interaction type was scored from 40 to 80 cotyledons per experiment. Graphs represent the mean and ±SE from three independent experiments.

Fate of Superoxide at HR Margins and RCD Sites Is Different

Because provision of $O_2^{-}\cdot$ is both necessary and sufficient to induce RCD in *lsd1* plants (Jabs et al., 1996), we addressed directly whether *EDS1*, *PAD4*, and *NDR1* act as signaling intermediates between superoxide and the *LSD1*-controlled cell death pathway. We elicited superoxide production by applying a discrete spot of rose bengal (RB) to leaves. In the presence of light, RB generates singlet oxygen that reduces to superoxide, which is rapidly dismutated to the more stable H_2O_2 (Knox and Dodge, 1984; Baker and Orlandi, 1995). RB-induced plant cell death was confined to the application site in wild-type Ws-0 leaves, but it induced RCD in *lsd1* leaves, as shown in trypan blue–stained leaves at 3 DPI (Figure 5A). We assessed H_2O_2 accumulation over a time course (3 to 48 hr) of RB treatment. Within 3 hr, we observed intense DAB and trypan blue staining in the area of RB application (Figure 5A). From 27 hr onward, cell death foci were fixed in Ws-0 but expanded in *lsd1* (Figure 5A). In several independent experiments, RB treatments of wild-type, *eds1*/*lsd1*, *pad4*/*lsd1*, and *ndr1*/*lsd1* plants failed to elicit RCD (Table 1). The same responses were observed in leaves of all genotypes infiltrated with a xanthine/xanthine oxidase superoxide-generating system that was previously shown to induce lesions in *lsd1* (Jabs et al., 1996; data not shown).

In the earlier analysis by Jabs et al. (1996), superoxide accumulation was observed in live plant cells bordering the RCD lesions of *lsd1* leaves. We expected to see DAB precipitation at the leading margins of *lsd1* lesions that would be generated upon dismutation of $O_2^{-}\cdot$ to H_2O_2. However, there was no detectable H_2O_2 accumulation associated with *lsd1* RCD lesions after either RB-induced cell death (Figure 5A) or *Pp* inoculation (Figure 5B). Superoxide production, measured by nitroblue tetrazolium (NBT) staining, was not detected at any point associated with the *Pp*-induced HR or RB-induced cell death (data not shown). Superoxide accu-

mulation, however, was observed at the boundaries of developing lesions in *lsd1*, confirming previous results (Jabs et al., 1996; data not shown). These results suggest that the fate of superoxide generated as a component of the *R* gene–dependent HR is different from that produced in association with RCD in *lsd1*.

DISCUSSION

We demonstrate that *EDS1* and *PAD4*, two positive regulators of plant disease resistance, are essential components of a cell death control pathway regulated by *LSD1* in response to pathogen infection, BTH application, or provision of superoxide. Most importantly, the requirement for *EDS1* and *PAD4* during *lsd1* RCD is independent of their roles as mediators of various *R* gene functions. Additionally, *NDR1*, a third disease resistance signaling component, contributes to *lsd1* RCD during these responses.

EDS1 and *PAD4* Potentiate Plant Defense Signaling

Our most important conclusion is that the requirements for *EDS1* and *PAD4* in *lsd1* lesion formation are separable from their roles in localized *R* gene–mediated plant cell death, as shown in the model in Figure 6. For example, neither *EDS1*

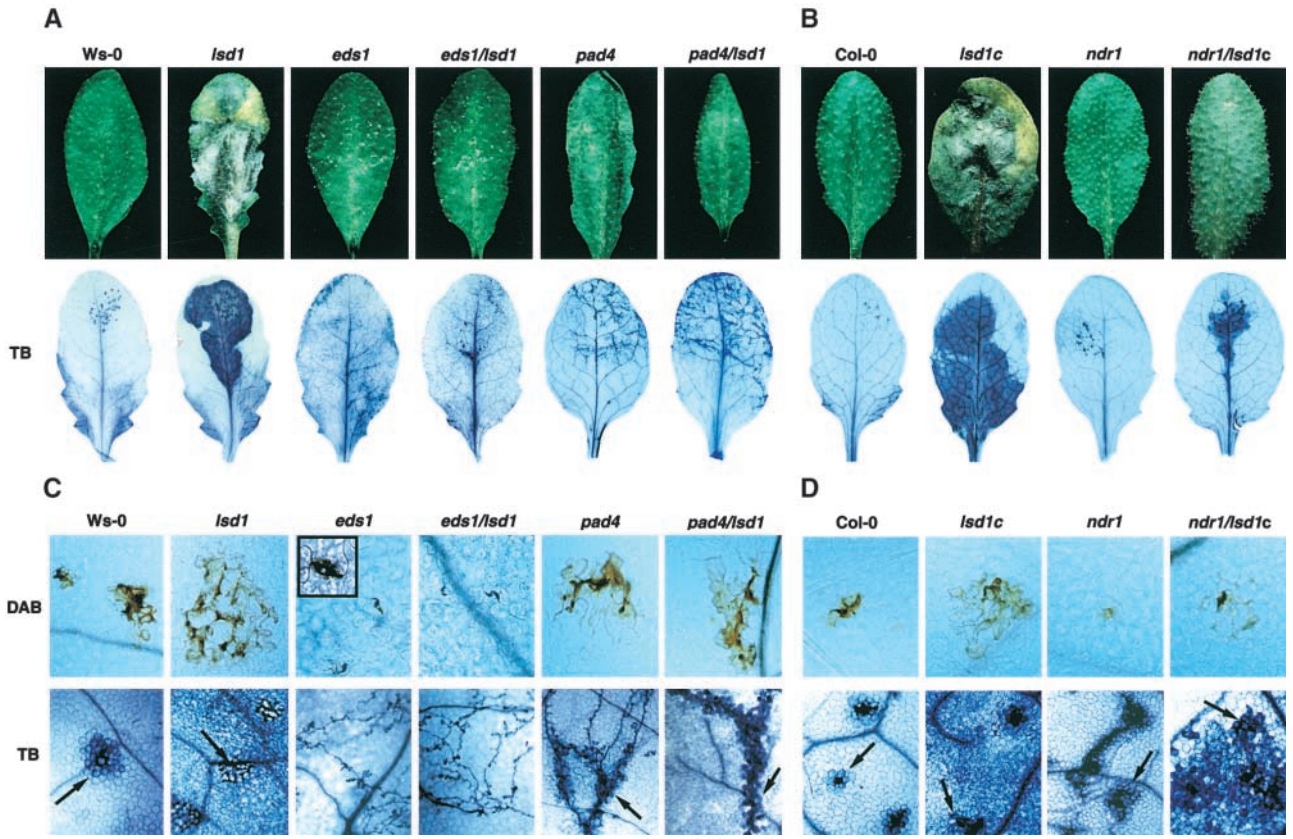

Figure 4. Disease Resistance and Runaway Plant Cell Death Phenotypes in Adult Leaves after *Pp* Inoculation.

Leaves of 4-week-old plants from wild-type, single mutant, and double mutant Ws-0 (**[A]** and **[C]**) and Col-0 (**[B]** and **[D]**) lines were inoculated by placing a 10-μL droplet of *Pp* spores on the top half of each leaf. Pp isolates Noco2 and Emoy2 (or Emwa1) were used for Ws-0 (**[A]** and **[C]**) and Col-0 (**[B]** and **[D]**) accession lines, respectively. Macroscopic phenotypes and corresponding trypan blue (TB) staining of plant–pathogen interaction sites are shown for whole leaves (**[A]** and **[B]**) and at ×200 magnification (**[C]** and **[D]**, bottom row) at 6 DPI. Hypersensitive plant cell death in trypan blue–stained leaves is marked with black arrows. Accumulation of H_2O_2 at interaction sites 32 hr after inoculation of leaves in the dark was measured using DAB and is shown at ×200 magnification (**[C]** and **[D]**, top row). The inset in the upper left corner of the *eds1* image at **(C)** shows an enlarged view of DAB staining restricted to the pathogen penetration site. Images are representative of four independent experiments using at least five leaves per genotype per experiment.

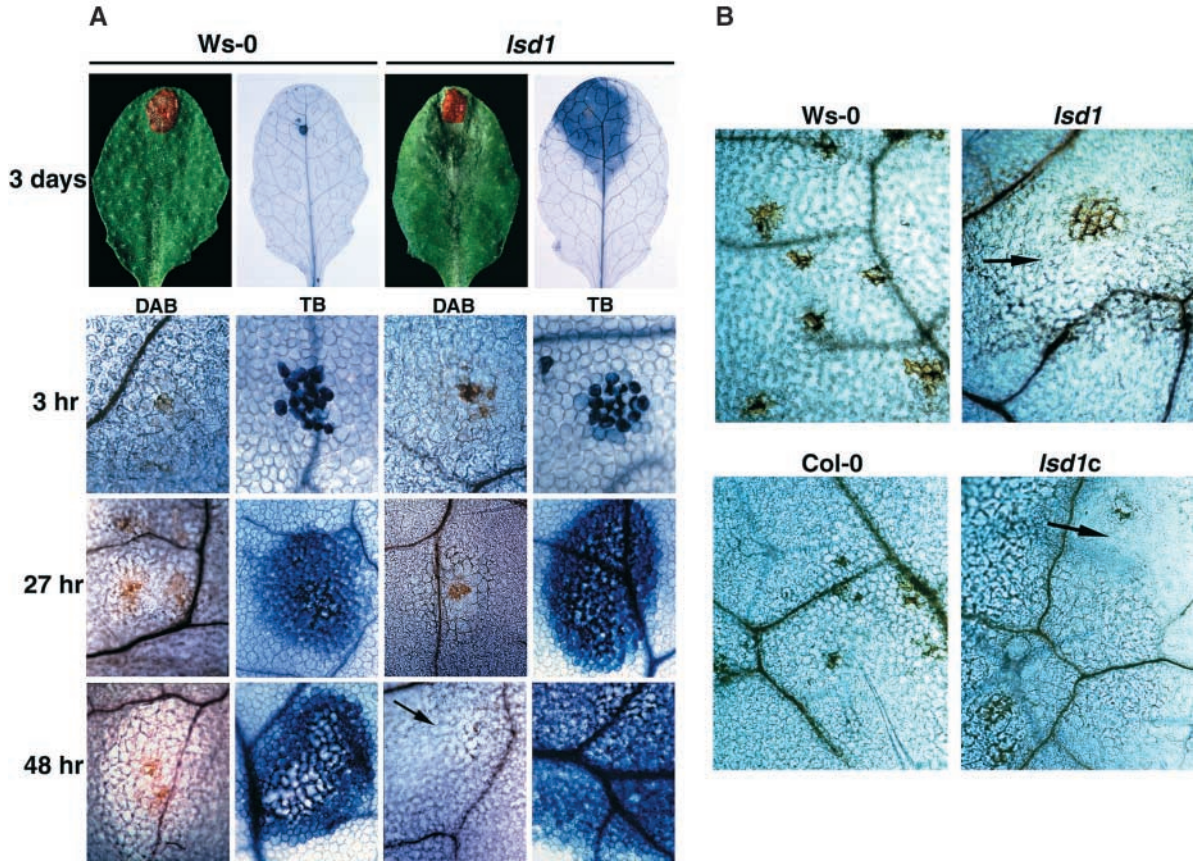

Figure 5. Localized H_2O_2 Accumulation in Wild-Type and *lsd1* Leaves after RB Application or *Pp* Infection.

(A) A single 5-μL spot of 20 mM RB was applied to leaves of 4-week-old plants. H_2O_2 accumulation was measured over 7 days by staining with DAB. Leaves also were stained with lactophenol–trypan blue (TB) to measure the extent of plant cell death. Localized RB application gives rise to local H_2O_2 accumulation associated with a discrete patch of dead plant cells. In Ws-0, expansion of plant cell necrosis ceases by 27 hr, whereas in *lsd1*, the lesions expand. DAB-staining material is detected in the area of RB application but is not associated with the spreading RCD in *lsd1* (black arrows).

(B) Leaves of 4-week-old plants were inoculated with a 10-μL droplet of 5×10^4 spores/mL Noco2 (Ws-0 and *lsd1*) or Emoy2 (Col-0 and *lsd1c*). Leaves were photographed 5 days after treatment. H_2O_2 accumulation, measured by DAB staining, was detected at plant–pathogen interaction sites but was not associated with spreading lesions, seen here as clear, unstructured cells (black arrows). Images are representative of three independent experiments using eight leaves per genotype per time point.

nor *PAD4* function in *RPM1* resistance, yet both are required for RCD after *RPM1* stimulation in *lsd1*. *EDS1*, but not *PAD4*, is necessary for ROI production and HR after local *RPS4*- or *RPP1*-mediated pathogen recognition, yet both *EDS1* and *PAD4* are required for *lsd1* RCD in these responses. We suggest that the activities of *EDS1* and *PAD4* leading to lesion formation in *lsd1* are in defense signal potentiation, downstream or independent of the HR (Figure 6). The finding that *eds1* and *pad4* suppress *lsd1* RCD in response to applications of BTH, a functional mimic of the plant resistance signaling molecule SA, is consistent with this idea. Other studies have shown the involvement of SA in signal potentiation during local and systemic plant defenses (Shirasu et al., 1997; Delledonne et al., 1998; Klessig et al., 2000; Martinez et al., 2000). *EDS1* (Falk et al., 1999; Feys et al., 2001) and *PAD4* (Zhou et al., 1998; Jirage et al., 1999) operate upstream of SA accumulation during resistance responses in which they are required. In these contexts, their expression levels are enhanced by the application of SA, suggesting that *EDS1* and *PAD4* are regulated by SA-dependent positive feedback (Falk et al., 1999; Jirage et al., 1999; Feys et al., 2001). We suggest that the flux through this feedback is regulated by *LSD1* (Figure 6). Experiments are in progress to determine directly the role of SA and the SA response regulator *NPR1*/*NIM1* (Cao et al., 1997; Ryals et al., 1997) in *lsd1*-dependent RCD. Notably,

ndr1 did not significantly suppress lesioning in *lsd1* after BTH treatment. This indicates that, in contrast to *EDS1* and *PAD4*, *NDR1* is not essential for BTH signaling (and presumably SA signaling) in relation to *LSD1*-regulated plant cell death.

EDS1, *PAD4,* and *NDR1* Mediate ROI-Dependent Signaling

It was shown previously that RCD in *lsd1* plants can be triggered by superoxide furnished by local applications of xanthine and xanthine oxidase (Jabs et al., 1996). Also, superoxide accumulation preceded lesion formation in *lsd1* tissue and was detectable in cells bordering the developing lesion by specific NBT staining (Jabs et al., 1996). Thus, *LSD1* activity appears to monitor a superoxide-dependent signal. Here, we show that *EDS1* and *PAD4*, and interestingly also *NDR1*, mediate the ROI-derived signal leading to *lsd1* RCD. The most compelling evidence for this is the failure of the *eds1/lsd1*, *pad4/lsd1*, and *ndr1/lsd1c* plants to initiate spreading lesions after local provision of superoxide, supplied either by RB (Table 1) or xanthine/xanthine oxidase applications. These data imply that all three disease resistance regulators express this particular function in unchallenged cells.

The activities of *EDS1* and *PAD4* in ROI signaling leading to RCD, therefore, are genetically distinct from their roles during the oxidative burst associated with a pathogen-induced HR (Figure 6). This finding strengthens the notion that *EDS1* and *PAD4* have a second function operating downstream or independently of the HR. We postulate that this second function helps establish the signal normally required to initiate *lsd1* RCD. However, three observations suggest a different role for *NDR1* in ROI signaling. (1) *ndr1* attenuated the oxidative burst during the HR through *RPS4* or *RPP4*, whereas it enhanced the oxidative burst during the HR through *RPM1* pathogen recognition. (2) In all of these plant–pathogen combinations, *ndr1* diminished *lsd1* RCD. (3) *NDR1* is not required for *lsd1* lesions in response to BTH but is required for lesion development in response to ROI provision. These three points lead us to conclude that *NDR1* is important in regulating the local ROI status (Figure 6). Imbalances in this system are likely to affect the efficiency of the HR and consequent local signaling and probably drive RCD in *lsd1*. Recent studies reveal that the balance of ROI, most particularly $O_2^{-}\cdot$, H_2O_2, and NO, is crucial for the establishment of the HR (Delledonne et al., 1998, 2001; Klessig et al., 2000).

We propose that *EDS1* and *PAD4* are regulators of ROI- and SA-dependent signaling in a plant defense potentiation circuit. We suggest that *NDR1* is required more proximally for the control of ROI generation and the transduction of a ROI-derived signal at the initial interaction site. In this respect, it is interesting that *EDS1* and *PAD4*, but not *NDR1*, are components of a basal resistance pathway that limits the growth of virulent pathogens in the absence of plant cell death (Glazebrook et al., 1996; Parker et al., 1996; Reuber et al., 1998). It is conceivable that basal resistance is a reflection of the *EDS1* and *PAD4* resistance-potentiating activities demonstrated here. Recent analyses revealed a requirement for *EDS1* and *PAD4* in constitutive SA-dependent resistance pathways induced by the *cpr1* and *cpr6* mutations (Clarke et al., 2001; Jirage et al., 2001) that is also consistent with resistance-potentiating roles.

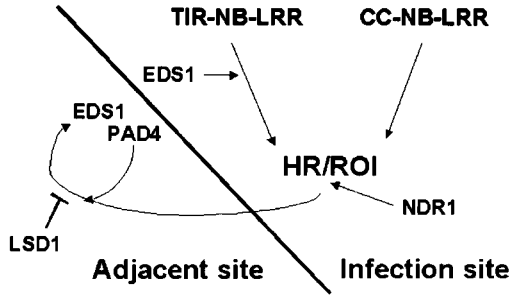

Figure 6. A Model Positioning EDS1, PAD4, and NDR1 in Relation to LSD1 in Plant Defenses.

As shown in the model, RCD in *lsd1* mutants is initiated in tissues adjacent to pathogen infection foci. The roles of EDS1, PAD4, and NDR1 in *lsd1* RCD are separable from events controlling the plant HR and its accompanying oxidative burst (ROI) that are elicited upon avirulent pathogen recognition. EDS1, but not PAD4, functions upstream of localized HR and ROI production in resistance conditioned by TIR-NB-LRR–type *R* genes. In contrast, resistance conditioned by CC-NB-LRR–type *R* genes operates independently of EDS1 or PAD4 but requires NDR1. Irrespective of the different requirements for EDS1 and PAD4 at initial infection foci, both components are essential for signal relay leading to RCD in *lsd1*. Because EDS1 and PAD4 are also necessary for *lsd1* RCD in response to the artificial provision of ROI or an active SA analog, we propose that EDS1 and PAD4 regulate a ROI/SA-dependent defense signal amplification loop. Flux through this loop is modulated by LSD1. NDR1 also is required for maximal lesion development in *lsd1* plants in response to pathogens. The data suggest that NDR1 acts more proximally by regulating ROI balance and transduction of ROI-dependent signals at infection sites (see Discussion for more details).

ROI Requirements Differ between the HR and *LSD1*-Controlled Plant Cell Death

Our analysis of ROI accumulation suggests that the nature of ROIs produced by cells undergoing the HR is different from that of ROIs associated with signaling from those cells, and monitored by *LSD1*. We detected superoxide, but not H_2O_2, in living cells bordering spreading *lsd1* lesions, as shown previously (Jabs et al., 1996). Our failure to observe H_2O_2 at these margins was surprising, because superoxide would be expected to dismutate to H_2O_2. LSD1 is required for the SA-dependent induction of antioxidant copper-zinc

superoxide dismutase (Cu-Zn SOD) (Kliebenstein et al., 1999) and potentially other antioxidant genes. Thus, a simple explanation is that there is no, or there is delayed, accumulation of Cu-Zn SOD in *lsd1* and hence no dismutation. This simple model is weakened by the unlikelihood that Cu-Zn SOD operates in the apoplast, where $O_2^{-}\cdot$ is first produced during the oxidative burst (Bolwell, 1999).

Another possibility is that superoxide produced by cells at HR margins, where *LSD1* is proposed to function, is converted to something other than H_2O_2. This could reflect an interplay between $O_2^{-}\cdot$ with other ROI molecules, SA, or antioxidant systems. In animal cells, superoxide can react with NO to produce peroxynitrite ($ONOO^{-}$), a highly reactive redox species that serves as a signal or as a cytotoxic agent, depending on its level and the availability of other redox molecules (Bonfoco et al., 1995; Lin et al., 1995). Delledonne et al. (2001) propose that $O_2^{-}\cdot$ production and its dismutation to H_2O_2 regulate a balance of H_2O_2/NO that, when disturbed, leads to HR. They argue against a direct role in cell killing for $ONOO^{-}$. An alternate explanation is that the H_2O_2 produced is locally unavailable for polymerization with DAB. This could be caused by changes in the cellular pH specific to these mutant backgrounds (DAB staining is effective only at pH values between 5.5 and 6.0; Thordal-Christensen et al., 1997) or by a surge of ROI scavenging enzymes (Vanacker et al., 2000). Cells are permeable to DAB and H_2O_2 (Thordal-Christensen et al., 1997), ruling out the possibility that H_2O_2 generated within the cell would be inaccessible for detection. Our data clearly suggest that the fate of superoxide produced in cells undergoing an HR is different from that generated locally during *lsd1* lesion development. This implies that signaling extending from infected cells is controlled differently than it is in the infected cells themselves.

Conversely, we observed H_2O_2, but not superoxide, at infection foci. Superoxide is an unstable redox molecule that rapidly dismutates enzymatically or nonenzymatically to H_2O_2 (Lamb and Dixon, 1997). Overwhelming evidence suggests that production of superoxide at the cell surface is the proximal event in the plant oxidative burst (Bolwell, 1999). However, other extracellular and intracellular mechanisms may contribute to ROI generation during the oxidative burst (Allan and Fluhr, 1997; Martinez et al., 1998; Bolwell, 1999). The transience of the oxidative burst and the inherent instability of superoxide may account for our failure to observe NBT-reactive material at infection sites or in cells supplied with superoxide by exogenous RB application. RB was applied onto the leaf surface and therefore would release superoxide into the plant apoplast that would be accessible to NBT (Baker and Orlandi, 1995).

Putative Signaling Functions of EDS1, PAD4, and LSD1

Our results draw an important genetic link between the disease resistance–promoting functions of *EDS1*, *PAD4*, and *NDR1* and the negative regulation of plant cell death exerted by *LSD1* (Figure 6), raising questions about the biochemical roles of these proteins in healthy and pathogen-challenged plants. *LSD1* encodes a zinc finger protein with similarity to the GATA-type family of transcription factors. EDS1 and PAD4 share homology with the catalytic domains of eukaryotic lipases (Falk et al., 1999; Jirage et al., 1999), although hydrolytic activities have not been demonstrated. It is possible, therefore, that they process ROI-activated signal intermediates spreading from infected to surrounding noninfected cells to perpetuate plant defense responses. In animals (Serhan et al., 1996; Stafforini et al., 1997) and plants (Farmer et al., 1998; Sanz et al., 1998; Rustérucci et al., 1999), activated fatty acids are important signaling molecules produced in response to certain pathogens and after wounding. Thus, EDS1 and PAD4 may potentiate resistance by processing ROI- and SA-activated molecules. The production of such molecules, whether lipid based or otherwise, would normally lead to cell death only if their levels passed a cell death control threshold. Obviously, in an *lsd1* null mutant, these levels need not be high to initiate RCD. The biochemical role of NDR1 also remains to be resolved, although its potential membrane association (Century et al., 1997) may be important in regulating cellular communication between external and internal redox systems. Elucidating the activities, cellular localization, and molecular associations of all of these signaling components should provide important insights into their precise functions in plant disease resistance.

METHODS

Plant Material and Cultivation

The origins of *eds1-1* (Parker et al., 1996) and *lsd1* (Dietrich et al., 1997) in accession Wassilewskija (Ws-0) have been described previously. The *pad4-5* T-DNA insertion mutant also was isolated in Ws-0 (Feys et al., 2001). The T-DNA is inserted 35 bp 5′ to the end of the single intron in the *PAD4* gene. The *ndr1-1* mutant line in accession Columbia (Col-0) (Century et al., 1997) was kindly provided by Dr. Brian Staskawicz (University of California, Berkeley). Seed were sown on low nutrient compost and grown in a chamber under a light period of 8 hr ($\sim$160 $\mu E \cdot m^{-2} \cdot sec^{-1}$) at 22°C and 65% relative humidity (RH).

F2 plants derived from selfed F1 plants were genotyped for the *lsd1* mutation by polymerase chain reaction (PCR) using a triple primer set (5′-ACCTAACAAAAAGAAAAGTGTGTGAGG-3′, 5′-ATAATAAACCCTACTAGCTCTAACAAG-3′, and 5′-CTGCTACTTTCATCCAAAC-3′). The wild-type *LSD1* allele produces a 940-bp product, whereas *lsd1* gives a 600-bp product. The Col-0 allele of *lsd1* (*lsd1c*) was constructed by introgressing the Ws-0 allele into a Col-0 line over seven generations and selecting for the mutant allele using the *lsd1* PCR described above. The *ndr1*/*lsd1c* double mutant was constructed by crossing *ndr1-1* plants with *lsd1c*, selfing the F1 plants, and genotyping the segregating F2 plants for the *lsd1* mutation (described above) and the *ndr1-1* mutation by using the primer set 5′-GGGACGGTTTCAATTCTGTGATAG-3′ and 5′-CGAGATTGC-

TCATTGCCATTGG-3′. The *eds1-1* mutation was detected in *eds1-1* × *lsd1* F2 plants using the primer set 5′-GGATAGAAGATGAATACA-AGCC-3′ and 5′-ACCTAAGGTTCAGGTATCTGT-3′. PCR products were digested for at least 4 hr with Mse1, and products were resolved on a 2% agarose gel. Cleavage of wild-type *EDS1* produces three visible bands of 280, 180, and 150 bp, whereas *eds1-1* gives visible products of 240, 180, and 150 bp. PCR-based selection of the *pad4-5* mutant allele in *pad4-5* × *lsd1* F2 plants was as described (Feys et al., 2001). In the initial characterizations of mutant phenotypes, we examined several independent mutant lines. All behaved similarly; hence, more detailed analyses were performed with one representative single and double mutant per genotype.

Pathogen Isolates and Growth Determinations

Peronospora parasitica (*Pp*) isolates Noco2, Emoy2, and Emwa1 were maintained on the genetically susceptible Arabidopsis accessions Col-0, Oystese, and Ws-0, respectively, as described previously (Dangl et al., 1992). To determine disease symptom development, *Pp* conidiospores were suspended in water (4×10^4 spores/mL) and sprayed onto 10-day-old (for cotyledon assays) or 4-week-old (for leaf assays) plants. Inoculated plants were kept under a sealed propagator lid to achieve high RH in a growth chamber at 19°C under an 8-hr light period (100 to 160 $\mu E \cdot m^{-2} \cdot sec^{-1}$). Alternatively, a 10-μL droplet of *Pp* conidiospores was placed on the leaf surface, and plants were incubated for up to 7 days under the same conditions as used for *Pp* growth assays.

Bacterial pathogen induction of runaway cell death (RCD) was measured by infiltrating suspensions (10^5 colony-forming units/mL) of *Pseudomonas syringae* pv tomato DC3000 expressing either *avrRps4* or *avrRpm1* into one side of the leaf using a 1.5-mL needleless syringe. Plants were inspected for disease symptoms and/or spreading lesion formation over 6 days under the same conditions as described for the growth assays. Hypersensitive response (HR) tests were performed using 5×10^7 colony-forming units/mL. Growth of *P. syringae* pv *tomato* DC3000 expressing *avrRps4* or *avrRpm1* in the various lines was determined by dip inoculation and subsequent growth analysis essentially as described (Innes et al., 1993) with modifications (P. Tornero and J.L. Dangl, unpublished data). Briefly, pots containing 2-week-old plants were immersed for 10 to 15 sec in a suspension containing 2.5×10^7 colony-forming units/mL ($OD_{600} = 0.05$) and Silwet (200 μL/L). Plants were kept under high humidity for 1 hr, after which time measurement zero was taken. At time 0 and 3 days, bacteria were extracted from the plant tissue and grown on selective agar plates to determine concentration.

Benzothiodiazole Induction of *lsd1* RCD

For chemical induction of RCD, leaves of 4-week-old plants were sprayed with 0.35 mM benzothiodiazole (BTH), which was provided as a gift from Syngenta (Research Triangle Park, NC). Plants were maintained under normal growth conditions and inspected for lesion development over 6 days.

Histochemical Analysis of Plant Cell Death and *Pp* Development

Plant cell necrosis induced by pathogen inoculation or chemical treatment, as well as the development of *Pp* mycelium inside cotyledon or leaf tissues, was monitored by staining with lactophenol–trypan blue and destaining in saturated chloral hydrate as described (Koch and Slusarenko, 1990). Material was mounted on a slide in 60% glycerol and examined using a light microscope (Axiophot; Zeiss, Jena, Germany). Excised leaves were manipulated in parallel with those used for detection of hydrogen peroxide (H_2O_2) and maintained under the same conditions (see below).

Histochemical Detection of H_2O_2 at Interaction Sites

Detection of H_2O_2 was by endogenous peroxidase-dependent in situ histochemical staining using 3,3-diaminobenzidine (DAB) in a protocol modified from Thordal-Christensen et al. (1997). Leaves of 4-week-old plants were inoculated with a 10-μL droplet of *Pp* conidiospores placed on the leaf surface. Leaves were then excised and supplied through the cut petiole with a solution of 1 mg/mL DAB for 8 hr in light (100 to 160 $\mu E \cdot m^{-2} \cdot sec^{-1}$) or in darkness under the same conditions used to determine *Pp* growth. Subsequently, the DAB solution was replaced with water, and leaves were maintained under the same conditions as before. For assessment of H_2O_2 accumulation at *P. syringae* infection sites, excised leaves were allowed to take up DAB solution for 8 hr and then were dipped in bacterial suspensions and incubated as described for the bacterial growth assays except that leaves were kept in the dark. At different times after pathogen inoculation, leaves were cleared for 5 min in boiling acetic acid/glycerol/ethanol (1:1:3 [v/v/v]) solution. Material was mounted on a slide in 60% glycerol and examined using a light microscope (Axiophot; Zeiss). H_2O_2 was detectable as reddish brown coloration.

Chemical Provision of ROI in Leaves

Rose bengal (4,5,6,7-tetrachloro-2′,4′,5′,7′-tetraiodofluorescein [RB]; Sigma) is an efficient singlet molecular oxygen ($^1O_2$) producer in aqueous solution (Knox and Dodge, 1984). $^1O_2$ gives rise to radical anion superoxide ($O_2^{-}\cdot$) and subsequently to H_2O_2. RB was applied as a droplet of 10 μL (20 mM solution) onto the surface of excised leaves of 4-week-old plants. These were placed in a growth chamber in the light (160 to 200 $\mu E \cdot m^{-2} \cdot sec^{-1}$) for at least 3 hr after RB treatment and maintained for several days under an 8-hr photoperiod at 19°C and 65% RH. Xanthine and xanthine oxidase coinfiltration in leaves of 4- or 5-week-old plants was used to generate superoxide, as described previously (Jabs et al., 1996). Infiltrated plants were maintained under normal plant growth conditions.

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